Phenotypic Heterogeneity and Cytotoxic Activity of Con A and IL-2-Stimulated Cultures of Mouse Thy-1$^+$ Epidermal Cells

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Short-term and long-term cultures of mouse Thy-1$^+$ epidermal cells (EC) were established in order to characterize their phenotypic and functional properties. Concanavalin A (Con A) and Interleukin 2 (IL-2) stimulated Thy-1$^+$ EC mediated non-MHC directed cytotoxicity preferentially against the NK-sensitive target, YAC-1 vs the NK-resistant target, P815; these cells also mediated antibody-dependent cell-mediated cytotoxicity (ADCC), indicating the presence of IgG-FcR on at least some of them. Freshly isolated Thy-1$^+$ EC failed to lyse YAC-1 targets; however, this activity was observed after 9 d of culture with Con A and IL-2. While dendritic Thy-1$^+$ EC, in vivo, do not express the T-cell markers, L3T4 and Lyt-2, short-term cultured cells displayed phenotypic heterogeneity with small but significant percentages of Lyt-2$^+$ and L3T4$^+$ cells appearing transiently. The phenotype of the effector cell(s), which mediates cytotoxic activity, was determined by utilizing flow cytometry to sort short-term cultured EC into positively and negatively stained populations. Cells which express L3T4, or which lack asialo GM_1, did not lyse YAC-1 targets; maximum cytotoxic activity was found within populations of cells which are asialo GM_1^+, Lyt-2$^-$, and asialo GM_1^+, Lyt-2$^+$. These studies indicate that Thy-1$^+$ cells derived from mouse epidermis when cultured in the presence of Con A and IL-2 have the capacity to generate a phenotypically heterogeneous population, some cells of which are capable of mediating cytotoxic activities. *J Invest Dermatol 91:62–68, 1988*

In 1983, bone marrow-derived, dendritic Thy-1$^+$ epidermal cells (EC) were observed in mouse skin [1–4]. It was observed that these cells were distinct from dendritic Langerhans cells in that they lacked Ia antigens, and cytoplasmic Birbeck granules; it was also apparent that they were not mature T lymphocytes as they failed to express other markers such as Lyt-1, Lyt-2, and L3T4 [2,5]. Interestingly, they did express a marker recognized by an unusual anti-Thy-1 antibody, 20-10-5S [6], which reacts with thymocytes, but not with mature, peripheral T lymphocytes in Thy-1.1 strains of mice, thus suggesting that these unusual epidermal cells might share properties with thymocytes [7]. The presence of large amounts of neutral glycolipid, asialo GM_1 [5,8], initially suggested that they might be related to natural killer (NK) cells [5,9,10]; however, it has become apparent that this marker is not specific for NK cells as it is also present on thymocytes [11] and alloimmune cytotoxic T lymphocytes [12].

We [7] and others [13] have reported that mouse EC proliferate in response to the T-cell mitogen, concanavalin A (Con A), and that Con A-induced proliferation is enhanced by the addition of interleukin 2 (IL-2) [7]. Using flow cytometry to purify Thy-1$^+$ cells, we demonstrated that the cells responsible for this proliferative response reside within the population of Thy-1$^+$ EC.

At the time these studies were initiated several hypotheses had been proposed for their lineage and possible immunologic functions, including thymocyte-like cells or pre-T lymphocytes [7], NK cells [5], cells involved in the suppressor cascade [14], or some other as yet uncharacterized and unique cell. Utilizing techniques previously described [7], we have established short-term and long-term cultures of epidermal-derived Thy-1$^+$ cells which have enabled us to characterize their phenotypic and functional properties. In this study, we demonstrate that Con A and IL-2-stimulated cultures of Thy-1$^+$ EC exhibit phenotypic heterogeneity, with significant numbers of Lyt-2$^+$ and L3T4$^+$ cells being present transiently. Cultured Thy-1$^+$ EC mediate cytolytic activity, which is non-MHC directed. The nature of this cytolytic activity and phenotypic heterogeneity, as well as the relationship of Thy-1$^+$ EC to cells of T lymphocyte or NK lineage is discussed.

MATERIALS AND METHODS

Mice Female CBA mice were purchased from Cumberland Laboratories (Cumberland, MD). Female BALB/c and A/J mice were

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Abbreviations:

ADCC: Antibody dependent cell-mediated cytotoxicity
CTL: Cytotoxic T lymphocyte
EC: Epidermal cell(s)
IgG-FcR: Receptors for the Fc portion of IgG
Interface EC: Epidermal cells recovered from Isolymph-medium interface
NK: Natural killer
SC: Spleen cells
10-10 IEC: long-term line of Con A and IL-2-stimulated interface EC
TCR: T-cell receptor

purchased from Jackson Laboratories (Bar Harbor, ME). All mice used were between 6 and 20 weeks of age.

Cell Preparations

Epidermal cells: EC enriched for Thy-1$^+$ EC were prepared as described previously [7,15]. Briefly, single cell suspensions were prepared from trypsin-disaggregated epidermal sheets and have been shown to contain 2%–4% Thy-1$^+$ cells and 2%–4% Ia$^+$ LC. Cell suspensions were layered over Isolymph (Gallard-Schlesinger Chemical MG. Corp., Carle Place, NY) and centrifuged at 23°C for 20 min. The Isolymph-medium interface was harvested (interface EC) and the cells washed and counted. Interface EC were routinely observed to contain 20%–60% Thy-1$^+$ cells (Thy-1$^+$ EC) and 10%–20% Ia$^+$ Langerhans cells [7,15].

Spleen cell (SC), lymph node (LN), and thymocyte suspensions: Spleens, lymph nodes, or thymuses were removed from mice and minced with scissors and forceps in Hank's Balanced Salt Solution supplemented with 10 mM HEPES, 10% fetal calf serum (FCS), and penicillin (100 U/ml)/streptomycin (100 μg/ml) (GIBCO, Grand Island, NY). Cells were filtered and washed twice prior to further use. In some experiments SC were treated with 0.3% trypsin (Type XI, Sigma Chemical Co., St. Louis, MO) in GNK solution (0.1% glucose, 0.88% NaCl, and 0.04% KCl) or in GNK solution alone, pH 7.6 for 10 min at 37°C.

Culture Conditions Culture conditions for interface EC have been described previously [7]. Two to 4×10^4 interface EC were cultured in 96-well U-bottom microtiter plates (Corning Glassworks, Corning, NY) at 37°C (5% CO_2) in complete RPMI [RPMI 1640 supplemented with 10% FCS, 1% non-essential amino acids, 25 mM HEPES, 2×10^{-5} M L-glutamine, penicillin/streptomycin, 10^{-5} M Na pyruvate (GIBCO), 5×10^{-5} M 2-mercaptoethanol]. Con A (1.5 to 2.5 μg/ml) (Pharmacia Fine Chemicals, Piscataway, NY) and 5 to 10 U/ml of partially purified rat IL-2 (Collaborative Research, Lexington, MA), or recombinant human IL-2 (Amgen, Thousand Oaks, CA) were added to initiate cultures of interface EC. Indomethacin (1 μg/ml) (Merck, Sharp, & Dohme, Rahway, NJ) was always used when cultures were initiated because it has been shown to enhance proliferation of Thy-1$^+$ EC [7,13]. After 1 week of culture, wells were harvested, and cells were pooled. Cultures were split and fed IL-2 (5 to 10 U/ml) 2 to 3 times per week and restimulated with Con A (1 μg/ml) every 2 to 3 weeks.

^{51}Cr-release Assay To measure cytolytic activity, a standard 4-h ^{51}Cr-release Assay was used as described previously [16]. Briefly, 1-5 $\times 10^6$ target cells were labeled with 100–250 μCi $Na_2\,^{51}CrO_4$ (New England Nuclear, Boston, MA) for 1.5 h at 37°C in 5% CO_2. Labeled targets were washed three times and resuspended in complete RPMI. One to 5×10^3 targets in 0.1 ml were placed in wells of Microtest II U-bottom plates with varying numbers of effector cells to yield a final volume of 0.2 ml. Each ratio was plated in triplicate. Plates were spun at 1000 rpm for 2 min, to gently pellet cells allowing maximum contact of effectors and targets. After 4 h at 37°C (5% CO_2) plates were centrifuged at 200 $\times$ g for 5 min. One hundred microliters of supernatant were harvested from each well and ^{51}Cr radioactivity was counted in a Packard Prias Autogamma counter (Packard Instruments, Inc., Fullerton, CA). Spontaneous release of ^{51}Cr was defined as the amount released from targets plated in the absence of effector cells. Maximal release was determined by the amount of ^{51}Cr released from targets lysed with 25 μl of Zapoglobin (Coulter Diagnostics, Inc., Hialeah, FL). Spontaneous release ranged from 10%–30% of maximal release. Mean specific cytotoxicity was calculated as follows:

$$\text{Mean percentage cytotoxicity} = \frac{^{51}\text{Cr release (experimental-spontaneous)}}{^{51}\text{Cr release (maximal-spontaneous)}} \times 100$$

Target Cells The YAC-1 Moloney virus-induced T lymphoma (H-2^a) and the methylcholanthrene-induced mastocytoma of DBA/2 mice, P815 (H-2^d), were maintained in vitro in complete RPMI. In antibody-dependent cell-mediated cytotoxicity (ADCC) assays, P815 tumor cells were used as targets. After labeling with ^{51}Cr, P815 targets were incubated for 30 min with a 1:100 dilution of C3H anti-DBA/2 (anti-H-2^d) anti-serum (gift from F. Zuckerman), and then washed twice. Antibody-coated P815 were used as controls for spontaneous ^{51}Cr release.

To prepare lymphoblast targets, 10^7 LN cells from CBA, A/J, or Balb/c mice were cultured in 10 ml complete RPMI in 25-cm tissue culture flasks in the presence of Con A (3.5 μg/ml) at 37°C (5% CO_2). After 3 d, cells were harvested, washed, and centrifuged through Isolymph at 1200 rpm for 10 min (23°C). Lymphoblasts recovered from the Isolymph-medium interface were washed twice and resuspended in complete RPMI prior to labeling with ^{51}Cr.

Effector Cells SC suspensions were prepared as described above and erythrocytes were removed by hypotonic lysis. Interface EC were used after residence in tissue culture for varying periods of time (4 h to 10 mon). For use as controls, alloreactive cytotoxic T lymphocytes (CTL) were prepared from LN of CBA or Balb/c mice. Five $\times 10^5$ LN cells in 2.0 ml complete RPMI were cultured in 24-well plates with 5×10^5 irradiated allogeneic stimulator SC from A/J, Balb/c, or CBA mice to generate CBA anti-A, CBA anti-Balb, or Balb anti-CBA alloreactive CTL. After 6 d, recovered CTL were harvested, washed, and resuspended in complete RPMI for use as effector cells in ^{51}Cr-release assays.

Analysis and Purification of Thy-1$^+$ EC Subpopulations by Flow Cytometry Four monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA) and used at a 1:50 dilution: anti-Ia^k, anti-Ia^d (control for anti-Ia^k), anti-Thy-1.2, and anti-Lyt-2. Culture supernatant from hybridoma GK1.5, producing anti-L3T4 monoclonal antibody [17], was used undiluted. Anti-Thy-1.2 (mouse IgM), purchased from New England Nuclear was used at 1:1000 dilution for analysis or sorting of freshly isolated interface EC. Rabbit polyclonal anti-asialo GM_1 (Wako, Dallas, TX) was used at a dilution of 1:100 or 1:200. The following second antibodies were used: Fluorescein isothiocyanate (FITC)-conjugated F(ab')$_2$ mouse anti-rat IgG (1:50 dilution); FITC-conjugated F(ab')$_2$ goat anti-rat IgG (1:50 dilution) (Jackson Immunoresearch Laboratories, Avondale, PA); FITC-conjugated F(ab')$_2$ rabbit anti-mouse IgG (heavy and light chain) (1:50 dilution) (Zymed, South San Francisco, CA); and FITC-conjugated goat anti-rabbit (IgA, IgG, and IgM) diluted 1:200 (Cappel Laboratories, Cochranville, PA). Controls were incubated with either normal rat Ig, an irrelevant primary of identical isotype, or normal nonimmune rabbit serum followed by the appropriate FITC-conjugated second antibody. Methods for staining, analysis, and sorting of cells have been described previously [7]; all studies were conducted with an Ortho Diagnostic Cytofluorograph System 50-H, using a 2150 Computer System (Ortho Diagnostics, Westwood, MA).

RESULTS

Morphology and Phenotype of Short-term and Long-term Cell Lines The ability to culture Thy-1$^+$ EC after stimulation with Con A and IL-2 [7] permitted the establishment of short-term (1–4 weeks) and long term (>4 weeks) cell lines as described in the "Methods" section. Morphologically, short-term cultured cells were indistinguishable from spleen cells cultured under the same conditions; i.e., small, angular to larger round cells (data not shown). Cells from the long-term line, 10-10 IEC, although initially similar to short-term cells, became large and granular; by electron microscopy 10-10 IEC contained numerous electron dense granules and a lobulated nucleus (data not shown). This large granular lymphocyte morphology is similar to what has been described both for purified NK cells [16] and CTL [18].

Freshly isolated interface EC obtained from the isolymph-medium interface are enriched for Thy-1$^+$ EC (20%–60%) and Langerhans' cells (LC) (8%–20%) [7,15]. Interface EC were cultured as described above with Con A and IL-2 and analyzed by flow cytometry at varying times for their phenotypic profiles. Table I demon-

Table I. Phenotypic analysis of cultured interface EC

Antibody	Mean % Positively Stained Cells ± S.D. at 1 week	2 weeks	≥ 4 weeks[a]
Anti-Thy-1.2	89 ± 4	95 ± 6	94 ± 7
Anti-Ia^k	8 ± 5	<1	ND
Anti-L3T4	4[b]	6 ± 6	0
Anti-Lyt-2	10 ± 11	18 ± 7	14 ± 19
Anti-asialo GM_1	11	33 ± 11	75 ± 3

[a] Cultures of greater than 4-weeks old included the long-term line 10-10 IEC.
[b] Represents data from one experiment. All other data represent the mean ± SEM of 2 to 7 analyses.

strates that phenotypic heterogeneity was observed within these short-term cultures of interface EC. After 1 week of culture a mean of 89% of cells were Thy-1^+ and 8% were Ia^+. Ia^+ cells were presumably LC, because the only cells that expressed Ia antigens were Thy-1^- dendritic cells as determined by immunoperoxidase labeling of cytospin preps of cultured cells (Bergstresser, unpublished observations). After 2 weeks of culture virtually all cells (95%) expressed Thy-1, and no Ia^+ cells were present. Low percentages of L3T4$^+$ cells were observed in the first and second weeks of culture, but they were undetectable in cultures greater than or equal to 4 weeks old. Lyt-2 was invariably expressed by a low percentage of cells from 2-week cultures; however, after 4 weeks we were able to detect small percentages of Lyt-2^+ cells in some, but not other, experiments. The combined percentage of L3T4$^+$ and Lyt-2^+ cells at 2 weeks accounted for less than or equal to 26% of the total number of Thy-1^+ cells. This was in contrast to Con A and IL-2-stimulated 2-week cultures of peripheral lymph node or spleen cells, in which the combined percentage of Lyt-2^+ and L3T4$^+$ cells was approximately equal to the total percentage of Thy-1^+ cells (data not shown).

Anti-asialo GM_1 stains Thy-1^+ dendritic EC brightly in epidermal whole mounts [3,5]; however, it was present on only 11% of cells after 1 week of culture. In fluorescence histograms of 1-week-old cultured cells stained with anti-asialo GM_1 the peak of the curve was shifted slightly to the right compared with the negative control, suggesting that the majority of cells were stained, but with low intensity (data not shown). With increasing time in culture the intensity of staining increased, and therefore, the percentage of positively stained cells also increased. Thus, histograms of anti-asialo GM_1 staining (Fig 1*b*) of Thy-1^+ 10-10 IEC (Fig 1*b*) and cells derived from cultures greater than 4 weeks old (data not shown) demonstrate a shift to the right in their staining profile suggesting that all the cells are positive. In this experiment 75% of the cells were positively stained above background staining, and 25% were weakly stained and considered negative. These data demonstrate that at least a subset of the 20%–60% Thy-1^+ EC derived from the interface EC population expand in culture after stimulation with Con A and IL-2 to yield a population that is virtually all (≥95%) Thy-1^+. These Thy-1^+ EC give rise to a heterogeneous population,

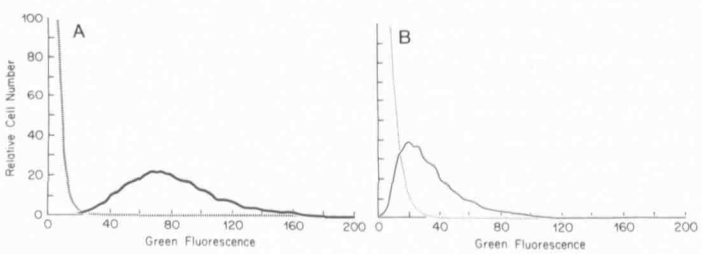

Figure 1. Phenotypic analysis of the long-term Thy-1^+ EC line, 10-10 IEC. *A*, cultured cells were stained with anti-Thy-1.2 *(solid line)* or control rat immunoglobulin *(dashed line)* followed by FITC-anti-rat IgG. *B*, cultured cells were stained with anti-asialo GM_1 *(solid line)* or with normal rabbit serum *(dashed line)* followed by FITC-goat anti-rabbit.

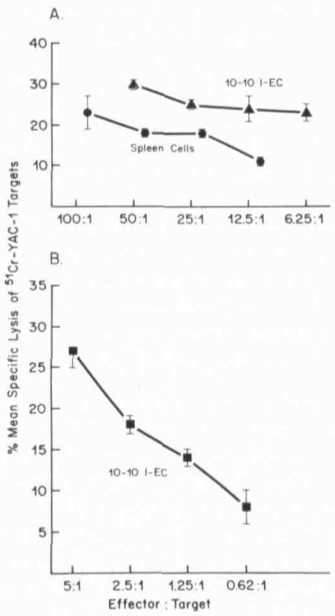

Figure 2. Cytolytic activity mediated by long-term cell line, 10-10 IEC. *a*, freshly isolated spleen cells or 10-10 IEC were plated in triplicate with ^{51}Cr-labeled YAC-1 targets at varying E:T ratios for 4 h. *b*, in a separate experiment cytotoxic activity was assessed at lower E:T ratios. Data is expressed as percent mean specific lysis ± SEM.

including cells which are Thy-1^+, L3T4$^+$; Thy-1^+, Lyt-2^+; Thy-1^+, L3T4$^-$, Lyt-2^-; and Thy-1^+, asialo GM_1^+, L3T4$^-$, Lyt-2^-.

Cytolytic Activity is Mediated by Cultured Thy-1^+ EC Because the morphology and phenotype (i.e., large granular cells expressing Thy-1 and asialo GM_1 antigens) of cultured Thy-1^+ EC resembled that described for NK cells [11], we tested these cultured cells for their capacities to lyse the NK-sensitive tumor target, YAC-1. Figure 2*a* shows the results of a representative experiment demonstrating that the long-term Thy-1^+ line, 10-10 IEC, lysed YAC-1 targets better than freshly isolated spleen cells at all effector to target (E:T) ratios. Figure 2*b* demonstrates that decreasing cytotoxic activity was observed at very low E:T ratios.

Figure 3 demonstrates that short-term cultured Thy-1^+ EC also mediated substantial lysis against YAC-1 targets that was comparable to that mediated by 10-10 IEC. Short-term cultured cells did not lyse the relatively NK-resistant tumor target, P815; however, the long-term line, 10-10 IEC (after 6 months in culture), did mediate significant lysis of P815. "Broadening" of target specificity has been reported for purified NK cells after culture [19], as well as for cultured T lymphocytes which mediate non-MHC directed cytotoxicity [18].

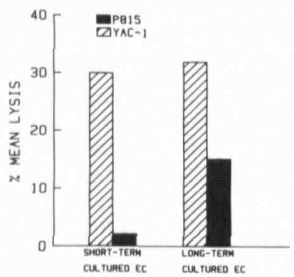

Figure 3. Target specificity of cultured EC against NK-sensitive YAC-1 and NK-resistant P815. EC derived from the long-term line, 10-10 IEC, or from short-term (9 d)-cultured Con A and IL-2-stimulated interface EC were plated in triplicate with ^{51}Cr-labeled targets at an E:T of 100:1. Data are from a representative experiment (n = 3); SEM in this experiment was less than or equal to 3%.

Table II. Cytolytic Activity Mediated by Long-Term Line, 10-10 IEC, and Allospecific CTL Against Lymphoblast Targets.

Effectors	% Lysis of ^{51}Cr-labeled lymphoblasts[a] at E:T of 25:1 A (H-2^a)	Balb (H-2^d)	CBA (H-2^k)
CBA anti-A CTL[b]	23 ± 5	ND[c]	ND
CBA anti-Balb CTL	ND	10 ± 3	ND
Balb anti-CBA CTL	ND	ND	31 ± 1
10-10 IEC	0	3 ± 0	0

[a] Lymphocytes from CBA, A/J, and Balb/c mice were stimulated with Con A (3.5 μg/ml) for 3 d. ^{51}Cr-labeled lymphoblasts were used as targets.

[b] Allospecific cytotoxic T lymphocytes (CTL) were prepared from LN of CBA and Balb/c mice in a mixed lymphocyte culture using irradiated allogeneic SC from A/J, Balb, and CBA mice to generate the listed CTL.

[c] Not tested.

To determine whether cytotoxic activity against YAC-1 was MHC-directed or was due to polyclonal activation of T lymphocytes, 10-10-IEC was tested for cytotoxic activity against a panel of allogeneic and syngeneic Con A-stimulated lymphoblast targets. Table II demonstrates that 10-10 IEC failed to lyse A/J or CBA lymphoblast targets, and had minimal, if any, activity against Balb/c lymphoblasts, yet these targets were capable of being lysed by the appropriate alloreactive CTL (lines 1 to 3). It is important to note that 10-10 IEC did not lyse A/J lymphoblasts which are H-2 (H-2^a) identical with YAC-1. Thus, lysis of YAC-1 by cultured interface EC is non-MHC directed.

Short-term cultured EC failed to lyse untreated P815; however, they mediated substantial lysis of anti-H-2^d-coated P815 targets (data not shown). This suggests that Con A and IL-2-stimulated interface EC contain cells which are capable of mediating ADCC via IgG-FcR, a function classically associated with NK cells which express receptors for the Fc portion of IgG (IgG-FcR) [20].

Freshly Isolated EC Fail to Mediate Cytolytic Activity against YAC-1 Tumor Targets, but Acquire This Activity after Short-term Culture Trypsinization is required to prepare interface EC. Trypsin-treatment of SC acutely diminishes the ability of splenic NK cells to bind to and lyse their targets; however, these activities return after 4 h of culture [21]. To determine whether freshly isolated Thy-1$^+$ EC mediate cytolytic activity against YAC-1 targets, freshly isolated CBA interface EC, and, as controls, SC which had been treated either with trypsin (in a similar manner as EC) or with buffer, were tested for their ability to lyse YAC-1 targets. Prior to assay cells were cultured for 4 h in complete medium with α/β-interferon (Lee Biomolecular Research, Inc., San Diego, CA) in an attempt to augment cytolytic activity [22,23]. Figure 4 reveals that both trypsin-treated and buffer-treated SC effectively lysed YAC-1 targets; by contrast, interface EC failed to exhibit cytolytic activity. Interface EC cultured for an additional 20 h with α/β-interferon and then tested in a 4-h assay still exhibited no cytolytic activity (data not shown).

The lack of cytolytic activity in freshly isolated EC was not due to the presence of other cellular inhibitors, because flow cytometry-purified Thy-1$^+$ EC (99% Thy-1$^+$) cultured for 20 h failed to lyse YAC-1 (data not shown). Incubation of sorted cells with α/β-interferon with or without IL-2 still failed to mediate cytotoxic activity. Furthermore, freshly isolated interface EC co-incubated with splenic NK cells had no inhibitory effect on the ability of NK cells to lyse YAC-1 targets (data not shown).

To determine when cytolytic activity appeared, cultured interface EC were tested at various times after culture. Figure 5 demonstrates that EC cultured for 3 d mediated minimal lysis of YAC-1; however, by 9 d of culture EC demonstrated substantial lytic activity, approaching that of the long-term line 10-10 IEC.

These data demonstrate that while freshly isolated splenic NK cells lyse YAC-1 targets, freshly isolated EC do not. The failure of EC to exhibit this activity cannot be attributed to trypsin-treatment or to the presence of inhibitors of NK-like activity. Cytotoxic activity, however, emerges after short-term culture with Con A and IL-2.

Phenotype of Short-term Cultured Thy-1$^+$ EC Which Exhibit Cytolytic Activity Against YAC-1 Interface EC cultured with Con A and IL-2 for 2 weeks were incubated with either anti-L3T4, anti-Lyt-2, anti-asialo GM_1, or a cocktail of anti-Lyt-2 and anti-asialo GM_1; controls were stained with normal rat IgG and/or nonimmune rabbit serum followed by the appropriate FITC-conjugated secondary reagent(s). Dead cells were gated out on the basis of forward and side scatter. Live cells from each group were sorted into either positively or negatively stained populations based on the control stains. The histogram profiles of anti-asialo GM_1 stained cells resembled Fig 1*b*. The sorted asialo GM_1-negative population therefore contained weakly positive and truly negative cells. The abilities of sorted positive and negative, as well as stained but unsorted populations, to exhibit cytolytic activity was assayed using YAC-1 targets. Similar results were observed in five experiments, with the results of a representative experiment shown in Table III.

L3T4$^+$ cells comprised 13% of the unsorted population (line 1). After sorting to 92% purity, L3T4$^+$ cells mediated essentially no lytic activity (line 2) against YAC-1 compared with stained, unsorted cells (line 1); all cytolytic activity remained within the L3T4$^-$ population (line 3). Lyt-2$^+$ cells comprised 19% of the unsorted population (line 4). After sorting, the positive population contained 87% Lyt-2$^+$ cells (line 5) and the negative population contained less than 1% Lyt-2$^+$ cells (line 6). Both the Lyt-2$^+$ and the Lyt-2$^-$ sorted populations lysed YAC-1 targets. By contrast, sorted asialo GM_1 negative cells containing only 3% asialo GM_1^+ cells were substantially depleted of cytolytic activity (line 9) compared with the unsorted population which contained 50% asialo GM_1^+ EC (line 7)

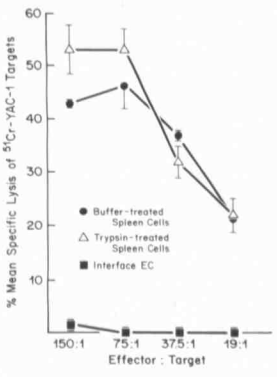

Figure 4. Cytolytic activity of freshly isolated interface EC and spleen cells against YAC-1 targets. Freshly isolated EC, and, as controls, spleen cells treated with trypsin, and spleen cells treated with buffer, were cultured for 4 h in complete RPMI in the presence of α/β-interferon. Cells were then plated in triplicate at varying E:T ratios with ^{51}Cr-labeled YAC-1 targets in a 4-h ^{51}Cr-release assay.

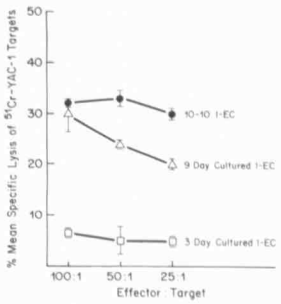

Figure 5. Appearance of cytolytic activity within cultures of interface EC. Cultures of Con A and IL-2-stimulated interface EC were harvested and pooled after 3 or 9 d of culture and tested for cytolytic activity against ^{51}Cr-labeled YAC-1 targets. Long-term line, 10-10 IEC, was used as a positive control.

Table III. Cytolytic Activity Mediated by FC-Sorted Populations of Cultured Thy-1$^+$ EC.

Antibody Pretreatment of Effectors	Effector[a] Populations	% Positively[b] Stained	% Lysis of ^{51}Cr-YAC-1 at E:T of 10:1	5:1	2.5:1
Anti-L3T4	Unsorted	13%	46 ± 1	30 ± 2	16 ± 3
	Positives	92%	6 ± 1	4 ± 1	2 ± 1
	Negatives	<1%	42 ± 3	30 ± 1	18 ± 3
Anti-Lyt-2	Unsorted	19%	40 ± 2	27 ± 1	18 ± 2
	Positives	87%	60 ± 4	33 ± 2	14 ± 1
	Negatives	<1%	41 ± 2	27 ± 1	17 ± 3
Anti-asialo GM_1	Unsorted	50%	54 ± 2	41 ± 3	32 ± 2
	Positives	88%	50 ± 4	26 ± 1	15 ± 1
	Negatives	3%	14 ± 1	6 ± 1	4 ± 1
Anti-Lyt-2 and Anti-asialo GM_1	Unsorted	51%	43 ± 3	27 ± 2	23 ± 6
	Positives	90%	40 ± 2	22 ± 2	12 ± 1
	Negatives	3%	2 ± 1	0	0

[a] Effector cells used: i) stained cells which were not sorted, i.e., unsorted; ii) cells which were sorted into positively stained populations; iii) cells which were sorted into negatively stained populations. Unstained, unsorted cultured EC were used as control effector cells in three experiments. The number of effectors used was based on the viable cell count (trypan blue) of each population just prior to plating in the cytotoxic assay. In 2 of 3 experiments lysis mediated by unstained, unsorted cells were equivalent to that mediated by stained, unsorted (for all antibodies). In this experiment stained, unsorted effectors mediated ≈ two-fold greater lysis of YAC than did unstained, unsorted effectors.

[b] The percentage of positively stained cells in the unsorted population was determined prior to sorting. Post-sort analysis revealed the percentage of cells positively stained in either the sorted positives or sorted negatives. The percentage of Thy-1$^+$ cells was not determined in this experiment because the percentage of Thy-1$^+$ cells in 2-week cultured Con A and IL-2-stimulated interface EC averaged 95% ± 6 in all previous experiments.

and with sorted positives which were 88% asialo GM_1^+ (line 8). Cultured Thy-1$^+$ EC were simultaneously stained with both anti-Lyt-2 and anti-asialo GM_1. If Lyt-2 and asialo GM_1 were present on separate populations of cells the percentages would be additive [e.g., 19% Lyt-2$^+$ cells (line 4) and 50% asialo GM_1^+ cells (line 7) would be 69%+ when stained with both anti-Lyt-2 and anti-asialo GM_1]. Line 11 demonstrates that only 51% of cells were stained with both these antibodies, and is equal to the percentage of cells stained with asialo GM_1 along (line 7). Thus, virtually all Lyt-2$^+$ cells were also asialo GM_1^+. By extrapolation, 19% of cells in this culture were Lyt-2$^+$, asialo GM_1^+, and 32% were Lyt-2$^-$, asialo GM_1^+.

When cells stained with both anti-Lyt-2 and anti-asialo GM_1 were sorted, the negative population containing only 3% Lyt-2$^+$, asialo GM_1^+ cells was virtually devoid of cytotoxic activity (line 12) with a 10–20-fold decrease in activity compared with the sorted positives (90% Lyt-2$^+$, asialo GM_1^+) (line 11) and the stained unsorted population (51% Lyt-2$^+$, asialo GM_1^+) (line 10). Thus, cytolytic activity appears to reside in two populations of asialo GM_1^+ cells: some of which are Lyt-2$^+$ and some of which are Lyt-2$^-$.

DISCUSSION

These experiments demonstrate that dendritic Thy-1$^+$ EC, which express asialo GM_1 but lack L3T4 and Lyt-2 in vivo, generate after short-term culture with Con A and IL-2 a phenotypically heterogeneous population with at least two subpopulations (e.g., asialo GM_1^+, Lyt-2$^+$ and asialo GM_1^+, Lyt-2$^-$), both of which are capable of mediating MHC-unrestricted cytotoxic activities. Although asialo GM_1 expression was low in young cultures, it increased steadily over time. By contrast, Lyt-2$^+$ and L3T4$^+$ cells appeared only transiently as a small percentage of cultured Thy-1$^+$ cells. These Lyt-2$^+$ and L3T4$^+$ cells may have been derived from small numbers of mature, peripheral T cells, present at the time of procurement, but this is not likely, because we have recently observed similar phenotypic and cytotoxic profiles in cultured Thy-1$^+$ EC derived from young (6-week old) T-cell-deficient nude mice [24]. In addition, Caughman and co-workers [13] have also observed phenotypic heterogeneity in cultured Thy-1$^+$ EC, noting either a Thy-1$^-$, asialo GM_1^+, L3T4$^-$ population or a Thy-1$^+$, asialo GM_1^-, L3T4$^+$ population. After comparing L3T4 expression between 48 and 72 h after trypsinization of interface EC with that of splenic T cells (L3T4 is trypsin sensitive), they suggested that at least the L3T4$^+$ population was not derived from contaminating peripheral T cells.

Cultured Thy-1$^+$ EC preferentially lysed the NK-sensitive target, YAC-1, compared with the relatively NK-resistant target, P815. This activity was not due to polyclonal activation of T cells because cultured Thy-1$^+$ EC failed to lyse a panel of allogeneic Con A-activated lymphoblasts. In addition, the failure to lyse lymphoblast targets which were H-2 (H-2^a)-identical with YAC-1 indicated that lysis of YAC-1 was not MHC-directed. The inability of short-term cultured Thy-1$^+$ EC to lyse P815 and syngeneic and allogeneic lymphoblasts indicated that these effectors were not lymphokine activated killer (LAK) cells, which lyse virtually all fresh or cultured tumor cells, as well as both syngeneic and allogeneic lymphoblasts [25]. Recently, we observed that bulk cultured Thy-1$^+$ EC as well as several Thy-1$^+$ EC clones mediate lectin-dependent cytotoxicity, a function classically ascribed to T lymphocytes [26,27]. Finally, it is now recognized that cytotoxicity against NK-sensitive targets can be mediated not only by NK cells (as defined by the lack of TCR gene rearrangements), but also by cytotoxic T lymphocytes (as defined by the expression of T3 and rearrangement of TCR genes), and this activity is best referred to as non-MHC-restricted cytotoxicity, rather than NK-like cytotoxicity [28]. Thus, our experiments, employing selected targets cannot differentiate further between these two mechanisms of killing.

Cytotoxicity against YAC-1 targets was mediated by both Lyt-2$^+$, asialo GM_1^+ cells, and by Lyt-2$^-$, asialo GM_1^+ cells, because cytotoxic activity was present in both Lyt-2$^+$ and Lyt-2$^-$ sorted populations, and was significantly depleted in the sorted asialo GM_1^-, and the Lyt-2$^-$, asialo GM_1^- populations. The failure of sorted asialo GM_1^+ cells (88% and 90% asialo GM_1^+, respectively) (Table III) to exhibit increased cytotoxic activity may be explained by the relatively high percentages of asialo GM_1^+ cells already present in the starting populations (50% and 51%, respectively) and by the possibility that sorted cells lose some activity due to excessive handling.

One explanation for the identification of two phenotypically identifiable cytotoxic effector populations (asialo GM_1^+, Lyt-2$^+$; asialo GM_1^+, Lyt-2$^-$) is that two distinct cells are present in epidermis: cells of T lymphocyte lineage and cells of NK lineage. Cultured Thy-1$^+$ EC mediated ADCC, indicating the presence of effector cells which express IgG-FcR. Moreover, Romani et al (5) have reported that a few dendritic Thy-1$^+$ EC in situ located around hair follicles also stain brightly with anti-FcR antibody. Recently we have identified IgG_{2a}Fc receptor α chain mRNA in 3-week cultured Thy-1$^+$ EC (unpublished observations). It is possible that these uncommon Thy-1$^+$, FcR$^+$ cells, which are present in freshly isolated EC preparations, undergo expansion during in vitro culture, and that they are responsible for ADCC. Although ADCC has been classically considered a function of IgG-FcR$^+$ NK cells [20], a subset of CD3$^+$ human T cells which mediate ADCC has recently been identified [29]. In mice, FcR for IgG and IgM have been

demonstrated on activated and resting T lymphocytes [30,31], but mouse T cells have not been reported to mediate ADCC. Ultimately, the question of whether heterogeneity among cultured Thy-1$^+$ EC represents disparate lineages (i.e., T3$^+$ T cell and T3$^-$ NK cell) should be resolved using a recently developed monoclonal antibody to the murine T3 determinant [32] as well as further analysis of the phenotypes and range of functional activities of recently established Thy-1$^+$ EC clones [33].

Phenotypic heterogeneity can also be attributed to the presence of two cells of T lymphocyte lineage; i.e., one that is Lyt-2$^-$, and one that differentiates to express Lyt-2. This hypothesis is supported by several observations. We have reported that dendritic Thy-1$^+$ EC are more closely related to T cells than to NK cells by their morphologic and functional absence in skin of C.B-17 *scid* mice, a mutant strain which has normal numbers of functional NK cells but lacks mature T and B cells [34]. Examination of long-term Thy-1$^+$ EC clones and lines has shown that these cells, unlike NK cells, rearrange and transcribe λ and δ TCR genes, and express functional λ and δ proteins associated with T3 on the cell surface [35–39]. Thus, Thy-1$^+$ EC resemble a subset of T cells found in mouse and human thymus and human peripheral blood which express T3 (mouse) or CD3 (human) associated with λ/δ TCR proteins, and lack the α/β TCR heterodimer [40–46]. These cells have a "double negative" phenotype; i.e, in mice T3$^+$, L3T4$^-$, Lyt-2$^-$ and in humans, CD3$^+$, CD4$^-$, CD8$^-$. At least some of these "double negative" thymocytes have the potential for limited proliferation and/or differentiation into Lyt-2$^+$ cells and L3T4$^+$ cells after in vivo thymic transplantation [47] and in vitro thymic organ culture [48].

The capacity for limited differentiation of these "double negative" cells into Lyt-2$^+$ cells parallels directly our experiments with Thy-1$^+$ EC. Hardt et al [49], recently reported rearrangement of the T cell receptor β-chain gene and induction of cytolytic function in day 14–15 fetal "double negative" thymocytes after culture with Con A, IL-2, and syngeneic feeder cells (conditions very similar to our own for Thy-1$^+$ EC). Lanier et al [46] have reported that a significant proportion of CD3$^+$, α/β^- peripheral blood lymphocytes (which were all "double negative" prior to culture) expressed CD8 (human Lyt-2 equivalent) after culture. Several reports demonstrate that T3$^+$, α/β^-, λ/δ^+ IL-2-dependent cell lines from human thymus and peripheral blood mediate non-MHC restricted cytotoxicity after culture with IL-2 [45,46,50]. Thus, not only do some thymocytes and peripheral blood lymphocytes that express a "double negative" phenotype have the capacity to differentiate into cells with phenotypes associated with more mature T cells, they also acquire cytotoxic activity during culture. In view of these similarities, it is possible that the L3T4$^+$ and Lyt-2$^+$ cells generated in our in vitro cultures are, in fact, derived from the Thy-1$^+$, Lyt-2$^-$, L3T4$^-$ EC, and that cytotoxicity is mediated by both Lyt-2$^-$ (perhaps less differentiated cells) and Lyt-2$^+$ (more differentiated cells) populations.

These studies have demonstrated that, with the appropriate stimulus, Thy-1$^+$ dendritic EC acquire the capacity to kill a target cell population via a non-MHC directed recognition mechanism and/or by ADCC. Although the importance of this function in vivo is not certain, our observation that purified hapten-conjugated Thy-1$^+$ EC, when given intravenously, down regulate a hapten-specific immune response [14], suggesting that these cells are involved in the regulation of other cells via a cytotoxic mechanism.

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